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Untargeted metabolic profiling reveals distinct patterns of thermal sensitivity in two related notothenioids

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ABSTRACT

Antarctic marine ectothermal animals may be affected more than temperate species by rising temperatures due to ongoing climate change. Their specialisation on stable cold temperatures makes them vulnerable to even small degrees of warming. Thus, addressing the impacts of warming on Antarctic organisms and identifying their potentially limited capacities to respond is of interest.

The objective of the study was to determine changes in metabolite profiles related to temperature acclimation. In a long-term experiment adult fish of two Antarctic sister species *Notothenia rossii* and *Notothenia coriiceps* were acclimated to 0 °C and 5 °C for three months. Impacts and indicators of acclimation at the cellular level were determined from metabolite profiles quantified in gill tissue extracts using nuclear magnetic resonance (NMR) spectroscopy. Furthermore, the metabolite profiles of the two con-generic species were compared.

NMR spectroscopy identified 37 metabolites that were present in each sample, but varied in their absolute concentration between species and between treatments. A decrease in amino acid levels indicated an increased amino acid catabolism after incubation to 5 °C. In addition, long term warming initiated shifts in organic osmolyte concentrations and modified membrane structure observed by altered levels of phospholipid compounds. Differences in the metabolite profile between the two notothenioid species can be related to their divergent lifestyles, especially their different rates of motor activity. Increased levels of the Krebs cycle intermediate succinate and a higher reduction of amino acid concentrations in warm-acclimated *N. rossii* showed that *N. rossii* is more affected by warming than *N. coriiceps*.

1. Introduction

Climate change is expected to produce the most dramatic environmental changes in the polar regions. Turner et al. reported an increase of the average sea surface temperature up to 2.8 °C/y around the Antarctic peninsula and its offshore islands for the period 1951–2000, presently followed by a cooling trend (Turner et al., 2005; Turner et al., 2016). According to the recent IPCC report water temperatures will reach temperatures above 5 °C around the Antarctic Peninsula by the end of this century (IPCC, 2014). Temperature affects the performance of organisms on all organisational levels, such as the kinetic energy of molecules, macromolecular stability, membrane properties, cellular processes and metabolic and physiological activities. In animals these changes result in an altered energy demand of cardiovascular, respiratory, gastrointestinal and excretory systems and therefore contribute to shape whole-organism performance (Guderley, 2004).

Temperature also determines the geographical distribution of species and their role within ecosystems. With ongoing climate change and its reported effects on organism and ecosystems, studies revealing the impacts of increasing temperature become more and more timely (Pörtner and Peck, 2010).

Warming has specific effects on the Antarctic fauna, in particular, on ectothermic organisms like fishes. The recent literature provides a profound overview of warming induced organismal changes in Antarctic fishes (see review of e.g. Pörtner et al., 2007), mainly under acute temperature changes. An increase in temperature is accompanied by elevated energy demand, visible in increased oxygen consumption rates and circulatory performance of Antarctic fishes during acute warming (Mark et al., 2002; Bilyk and DeVries, 2011). Longer incubation for 9 weeks to 4 °C showed a complete compensation of metabolic costs at the organismal level in *Trematomus bernacchii* (Sandersfeld, 2015). At the molecular level Windisch et al. (2011) could show that respiratory chain capacities increased accompanied by a shift from lipid to carbohydrate catabolism in the liver of the Antarctic eelpout *Pachycara brachycephalum* after long term acclimation to 5 °C. However, very little is known about the actual metabolic changes in

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Antarctic notothenioid fish after long term acclimation to warming. We therefore applied an untargeted NMR based metabolic profiling approach to gill tissue as an aerobic oxygen supplying organ, which sets whole organism performance. Recently, metabolomics was successfully applied to environmental studies on marine organisms (see reviews by Viant et al., 2003; Wu et al., 2008) and a couple of applications to marine fish can already be found in the present literature (e.g. Mannina et al., 2008; Liu et al., 2011; Picone et al., 2011). One of the main advantages using metabolic profiling is the integrative nature of this approach (Lenz and Wilson, 2007), as it provides an almost complete picture of the main concentration changes of metabolites under specific conditions. Thereby, the key metabolic responses to a specific stressor can be revealed (Dunn and Ellis, 2005).

In the present study, the thermal sensitivity and metabolic responses of two closely related Antarctic notothenioids were investigated after incubation for 3 months to 5 °C. The chosen temperature relates to the temperature expected in Antarctic surface waters around King George Island in the year 2100. Gill tissue extracts of the Antarctic rock cod *Nothothenia rossii* and the Antarctic yellow rock cod *Nothothenia coriiceps* were taken and analysed using untargeted NMR-based metabolic profiling.

Windisch et al. (2014) suggested a shift of an amino-acid and lipid-based metabolism to carbohydrate catabolism in the liver of *P. brachycephalum* after long term warming. Kullgren et al. (2013) found lower amounts of amino acids after long-term acclimation in the plasma of Atlantic salmon. In addition, new proteins need to be synthesised to compensate for damaged and non-functional proteins at higher temperatures and Antarctic species seem to have difficulties to synthesize proteins to full function (Peck, 2016). Therefore, our main focus laid on amino acids, especially those used for energy provision and involved in protein synthesis. Gill tissue was chosen for our study, as it is a high metabolic tissue that does not exhibit big metabolite storage capacities, such as muscle and liver for lipids (Sheridan, 1988). The investigation of the metabolic profile of the notothenioids provides an outline for metabolic shifts, which can serve as basis for further analyses. Investigating polar extracts placed a major focus on possible changes in the amino acid metabolism, the energy metabolism and cellular membrane structure. Furthermore, the comparison of the two con-generic notothenioid species *N. rossii* and *N. coriiceps* could point out possible distinctions of their acclimation capacities, which might be related to their divergent ecological backgrounds.

2. Materials and methods

2.1. Animal collection and acclimation experiment

Notothenia rossii and *Notothenia coriiceps* were caught in Potter Cove of King George Island (coordinates 62°14′S, 058°41′W) between February and March of 2010 using baited traps. The recorded water temperature during that time was 1.72 ± 0.13 °C and the salinity 34.03 ± 0.07 PSU.

All animals were transported to the Alfred-Wegener-Institute (AWI) in Bremerhaven in an aquarium container on board of RV Polarstern during VII/3–4. Afterwards the animals were kept in circular tanks with a volume of 2.6 m^3 under a 12 h light/12 h dark cycle in the polar aquarium facility of the institute until the experiment.

For the long-term acclimation experiment 5–7 individuals were placed in individual tanks for 3 months. For both species, *N. rossii* and *N. coriiceps*, fishes were randomly selected and kept each at temperatures of 0 ± 0.2 °C (control group) and 5 ± 0.2 °C. The salinity was maintained at ~32.5 PSU. Water quality was controlled conducting weekly ammonium, nitrite and nitrate tests (*Merck KGaA, Germany*). During the experimental period, the notothenioids were fed with thawed common cockles (*Cerastoderma edule*) twice a week.

After the acclimation experiment the experimental fish were anaesthetised with 0.5 g/l tricaine methano-sulfonate (MS222) and sacrificed by cutting the spinal cord. Gill tissues were dissected, flash frozen in liquid nitrogen and stored at − 80 °C until extraction.

The average standard length of the animals kept at 0 °C was 30 ± 2 cm for *N. rossii* (n = 12) and 32 ± 3 cm for *N. coriiceps* (n = 12). The average weight was 411 ± 75 g for *N. rossii* and 396 ± 90 g for *N. coriiceps*. For the animals acclimated to 5 °C the average standard length was 31 ± 4 cm at an average weight of 510 ± 166 g for *N. rossii* (n = 14) and 31 ± 2 cm at 560 ± 80 g for *N. coriiceps* (n = 12).

2.2. Sample preparation

Metabolites of fish gill tissue were analysed using Nuclear Magnetic Resonance (NMR) spectroscopy. Polar metabolites were extracted from tissues according to the two-step extraction protocol developed by Wu et al. (2008). The pre-weighed (~100 mg) frozen gill tissue samples were added to homogenisation tubes containing 400 µl ice-cold methanol and 125 µl ice-cold Milli-Q water. For homogenisation samples were minced in a bead-based *Precellys 24* (*Bertin Technologies, France*) for 1 cycle of 20 s at 6000 rpm and temperatures between 0 and 4 °C. The homogenisation step was done in groups of four samples to prevent tissue from thawing before homogenisation. 400 µl chloroform and 400 µl Milli-Q water were added to each homogenate before they were mixed thoroughly for 15 s. All samples were left on ice for 10 min to partition and thereafter centrifuged at 3000 g at 4 °C for 10 min. The upper, polar layer was transferred into a 2 ml Eppendorf cup and dried in a rotational vacuum concentrator (*RVC 2–18 HCl, Christ GmbH, Germany*) at room temperature overnight.

2.3. Untargeted NMR based metabolic profiling

Dried polar gill extracts were re-suspended in deuterated water (D_2O) to a final concentration of 1 g frozen gill tissue/ml solvent. The D_2O contained 0.05 wt% of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, sodium salt (TSP) (*Sigma Aldrich, St. Louis,* USA). TSP was used as chemical shift and quantification standard. For each sample 50 µl of the resuspended gill extracts were analysed.

The spectra were measured using an ultra-shielded vertical 9.4 T NMR spectrometer (*Avance III HD 400 WB, Bruker-BioSpin GmbH, Germany*) at a proton frequency of 400 MHz in combination with TOPSPIN 3.2 software (*TopSpin 3.2, Bruker-BioSpin GmbH, Germany*) and a high-resolution magic angle triple tuneable ^{1}H-^{31}P-^{13}C probe (HRMAS). All samples were analysed using one dimensional ^{1}H–HRMAS NMR spectroscopy with water presaturation at 21 °C. A set of four different NMR techniques were used for each sample as described in Schmidt et al. (2017). A. a standard one-pulse pulse sequence, B. a Call-Purcell-Meiboom-Gill (CPMG) sequence, C. a Nuclear Overhauser Effect Spectroscopy (NOESY) protocol and finally D. a J resolved (J-RES) sequence for signal assignment. The CPMG protocol was used for further analysis, measured with a relaxation delay of 4 s, a sweep width of 8803 Hz and 70 k data points. 32 scans were acquired for each spectrum.

The spectra were processed and analysed with *Chenomx NMR Suite 8.0* software (Chenomx Inc., *Canada*). Fourier-transformed spectra were multiplied with an exponential weighing function corresponding to a line-broadening between 0.5 and 1 Hz depending on spectrum quality. Before analysing them, all spectra were manually corrected for phase and baseline and referenced to TSP. The metabolite peaks of the processed spectra were analysed and assigned to their chemical shifts using the *Chenomx* database as a reference. The assigned peaks were compared and confirmed by chemical shift values in the literature (Fan, 1996; Gribbestad et al., 2005; Castejón et al., 2010). The concentration of the assigned metabolites was provided by the *Chenomx* software based on the concentration of the internal standard TSP. In addition to the one-dimensional ^{1}H–NMR spectrometry, a two-dimensional heteronuclear single quantum coherence measurement (^{1}H-^{13}C HSQC

NMR) of a *N. rossii* sample was taken and the resulting 2D spectrum was used to assist in assigning signals from the ^{1}H–NMR spectra (see Supplementary S1). The correlated resonances were identified using the online database Spectral Database for Organic Compounds (*SDBS, National Institute of Advanced Industrial Science and Technology, Japan*) together with data from the literature (see above).

To gain further information about the presence of phosphorus-containing metabolites in the tissue extract, a ^{31}P spectrum was measured with a relaxation delay of 8 s, a sweep width of 8012 Hz and 4 k data points. The peaks of the spectrum were assigned to metabolites using reference spectra from the literature (Canioni and Quistorff, 1994; Bock et al., 2001).

2.4. Statistical analysis

Metabolite concentrations in the gill tissue extracts of the different groups were analysed using univariate and multivariate statistical analysis. All obtained metabolite concentrations were related to a metabolite with a constant concentration for normalisation (Craig et al., 2006). The normalisation was performed on the adenosine signal, as the total adenosine concentration is expected to be constant, especially for long-term acclimation, just the number of bound phosphate groups varies (Hochachka and McClelland, 1997). In addition, a generalised log-transformation was applied to the normalised metabolite concentrations to stabilise the variance across the detected metabolite concentrations (Purohit et al., 2004).

For multivariate analysis, unsupervised principle component analysis (PCA) and supervised partial least-squares discriminant analysis (PLS-DA) were applied using *Metaboanalyst* software (*Metaboanalyst 3.0*; Xia and Wishart, 2016). Multivariate analysis was conducted for each pair of groups separately (both control groups, control vs. 5 °C acclimated group for each species and both groups acclimated to the elevated temperature). Results are presented reporting the scores of the principle components and showing a 2D score plot of the first two principle components. An example for a PCA and a PLS-DA loadings plot is presented in the Supplementary (S2).

Univariate analysis including parametric Student's *t*-test and non-parametric Mann-Whitney test was performed with *SigmaPlot* (*SigmaPlot 12.0,* Systat Software Inc.) to detect and validate the changes in the metabolite concentrations between the pairs of grouped samples investigated by multivariate analysis. The threshold for significance was a p-value < 0.05 for all tests.

3. Results

Fig. 1 presents an example of a typical ^{1}H–CPMG-NMR spectrum acquired from an aqueous gill tissue extract of *Notothenia rossii* obtained from this study. Signal changes of 41 signals were analysed from gill tissue. Four signals could not be assigned to a specific metabolite, leading to an overall identification of 37 metabolites. The concentration changes were used to analyse for a potential species and temperature dependent metabolic response. In Table 1 all identified metabolites are listed according to their metabolite classes and to their characteristic chemical shifts (only relevant chemical shifts are specified for each metabolite).

All spectra were dominated by the organic osmolytes trimethylamine-N-oxide (TMAO) and taurine, showing large peaks in the spectrum area between 3.25 and 3.30 ppm and 3.40–3.45 ppm. In general, osmolytes and amino acids represented the main metabolite groups detected in the aqueous extracts.

In the aliphatic region signals characterising the free amino acids leucine, isoleucine, valine, alanine, glutamine, glutamate and methionine were identified. The organic acids occurring in this region were lactate, succinate, citrate and acetate. A small singlet at 2.72 ppm was identified as dimethylamine (DMA).

Overlapping signals in the hydroxylic region resulted from various alpha amino acids, alcohols, polyols and saccharides. Characteristic patterns could be assigned to metabolites such as choline, O-phosphocholine (PC) and sn-glycero-3-phosphocholine (GPC). Peaks at 3.04 ppm and 3.94 ppm belonged to creatine and phosphocreatine. In addition, the distinct pattern of the polyol myo-inositol was detected. The amino acid glycine showed a recognisable singlet at 3.56 ppm and the osmolyte betaine at 3.87 ppm. Identified sugars in the hydroxylic region were glucose and UDP-glucose.

Signals obtained in the aromatic region were attributed to the aromatic amino acids phenylalanine, tyrosine and histidine. Other assigned metabolites were the nucleoside inosine and nucleotide derivates, such as adenosinetriphosphate (ATP), adenosinediphosphate (ADP), nicotinamide adenine dinucleotide (NADH) and nicotineamide adenine dinucleotide phosphate (NADPH). The two peaks at 8.19 ppm and 8.21 ppm indicated the presence of the purine derivative hypoxanthine.

The composition of assigned metabolites in the tissue extracts did not differ between the two notothenioid species, except for varying compound concentrations. Univariate and multivariate statistical analyses were used to reveal differences in metabolite concentrations in both Antarctic notothenioid species. The principal component analysis (PCA) of all gill tissue extract samples at 0 °C displays a homogeneous group with no detected outliers. 96.4% of the total variance in the metabolite concentrations was explained by the first principle component (PC1, Fig. 2A).

In order to address potential differences in metabolite concentrations between both groups a supervised partial least square discriminant analysis (PLS-DA) was performed. In the supervised PLS-DA a sharpening of the separation between the groups found in the PCA is performed by rotating the PCA components to obtain a maximum separation among classes. Other than the PCA the PLS-DA does take into account the correlation of the dependent and the independent variables. Fig. 2B presents the resulting score plot of the PLS-DA showing a significant separation of data from the two Antarctic notothenioid species under control conditions.

Table 2 lists the detected significant differences in metabolite concentrations based on a Student's *t*-test or a Mann-Whitney Rank Sum test as indicated in the table. The largest difference was found in the concentration of the osmolyte TMAO. *N. coriiceps* had almost twice the concentration found in *N. rossii*.

As a general pattern indicated by significant differences in the metabolite concentrations of the gill extracts, *N. coriiceps* had lower amounts of the free amino acids methionine, phenylalanine and tyrosine. All metabolites listed in the group of energy supplying compounds, such as creatine, phosphocreatine, citrate, glucose and NADH/NADPH did also exist in lower concentrations in this benthic species in comparison to *N. rossii*. Furthermore, metabolites involved in phospholipid metabolism, such as choline and its derivative glycerophosphocholine, were present in higher concentrations in the pelagic swimmer *N. rossii* than in its sister species.

No general pattern could be observed for metabolites belonging to various metabolic groups or having an unknown function (listed as miscellaneous). The amount of the purine derivative inosine was higher in *N. coriiceps*, while acetate and sarcosine levels were lower.

3.1. Differences in metabolite concentrations after warm-acclimation

In general, no additional metabolites were found in the gill samples taken from fish after long-term acclimation to 5 °C compared to the control group. Nevertheless, metabolite concentrations varied in the metabolic profiles between the groups for both notothenioids.

As in the PCA analysis of the control groups, there were no outliers detected in the different groups (data not shown). For the *N. rossii* groups PC1 explains 95.9% of the variance and the PCA analysis for *N. coriiceps* provided evidence, that 97.3% of the variance can be explained by the first principle component, demonstrating again a decent

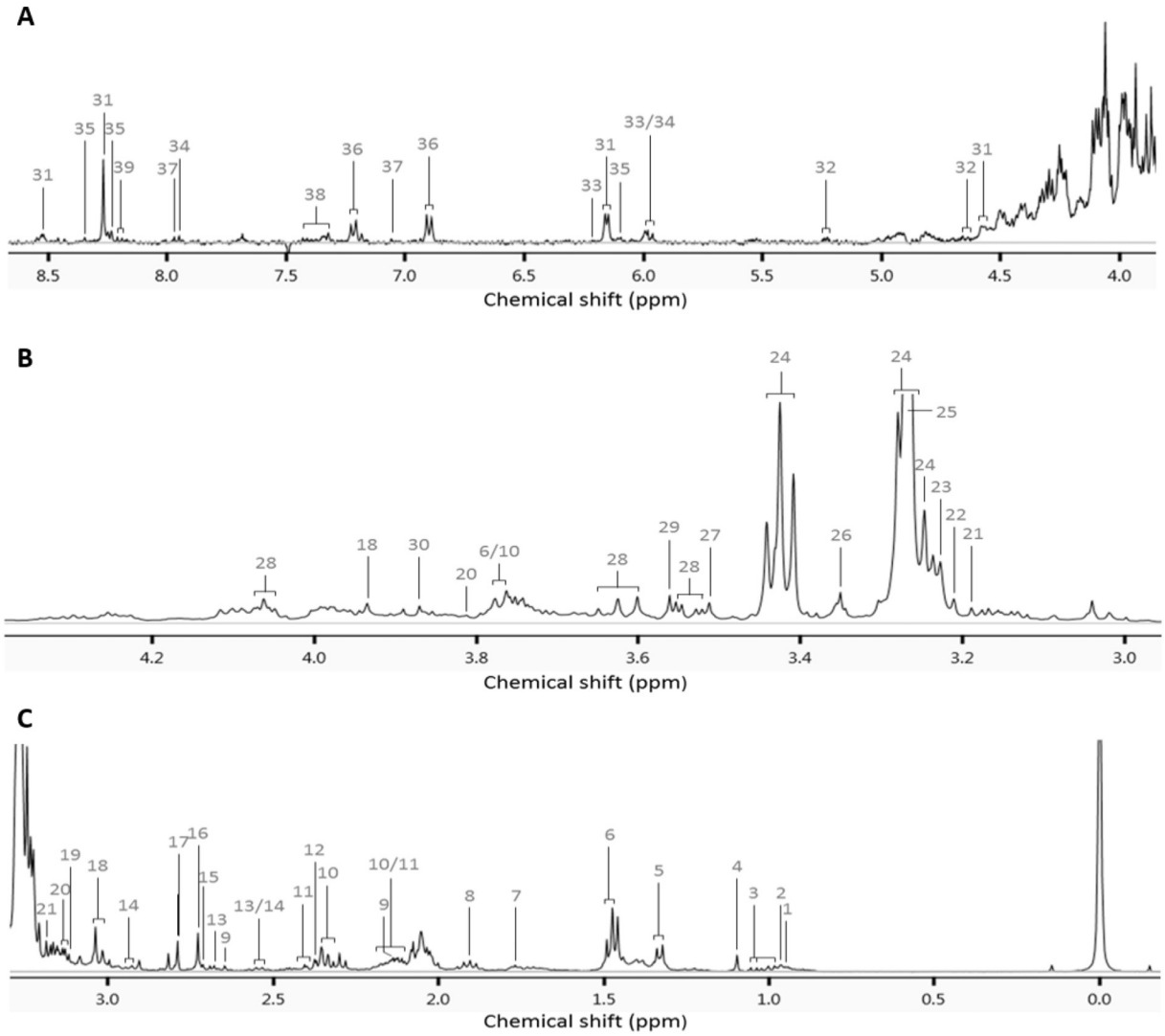

Fig. 1. Representative one dimensional 400 MHz CPMG ^{1}H–NMR spectra of gill tissue extract from *Notothenia rossii* acclimated to 0 °C with vertical expansion of the different regions (A-C).

Keys: 1 Isoleucine; 2 Leucine; 3 Valine; 4 Unknown 1; 5 Lactate; 6 Alanine; 7 Putrescine; 8 Acetate; 9 Methionine; 10 Glutamate; 11 Glutamine; 12 Succinate; 13 Citrate; 14 Glutathione; 15 Dimethylamine (DMA); 16 Sarcosine; 17 Unknown 2; 18 Creatine/Phosphocreatine; 19 Malonate; 20 Ethanolamine; 21 Choline; 22 Phosphocholine; 23 Glycerophosphocholine; 24 Taurine; 25 Trimethylamide-N-oxide (TMAO); 26 Unknown 3; 27 Unknown 4; 28 myo-Inositol; 29 Glycine; 30 Betaine; 31 ATP/ADP; 32 Glucose; 33 NADH/NADPH; 34 UDP-Glucose; 35 Inosine; 36 Tyrosine; 37 Histidine; 38 Phenylalanine; 39 Hypoxanthine.

Signals in the area between 4 and 4.5 ppm resulted from various alpha amino acids, alcohols, polyols and saccharides.

homogeneity of the analysed groups.

Fig. 3 displays the supervised PLS-DA between gill tissue samples of the two notothenioids under control and 5 °C. The PLS-DA was performed for each species separately as was the PCA analysis. The score plots of the PLS-DA depict a clear separation for both, *N. rossii* and *N. coriiceps* (Fig. 3). The ellipses corresponding to the confidence interval of 95% were clearly separated from control and the 5 °C acclimated group.

Table 3 summarises the significant changes in metabolite concentrations during thermal acclimation for each notothenioid species.

The main metabolite groups affected were amino acids, osmolytes and phospholipid metabolism related compounds. The largest changes are visible in the concentration of the osmolyte TMAO and the structural component myo-inositol, which both exist in high concentrations in the tissue, and of acetate (data not shown, see Supplementary material S3).

Osmolyte concentrations in gill tissues decreased after 5 °C incubation, a trend seen for all osmolytes. Significant changes were confirmed for TMAO and betaine in *N. rossii* and for TMAO and DMA in *N. coriiceps*. In addition, the concentration of the amino acid glycine was significantly decreased (see Table 3).

The concentrations of choline, GPC and myo-inositol, which are related to phospholipid metabolism, were significantly lower in the 5 °C acclimated groups than in the control group in *N. rossii.* The same was observed for choline and myo-inositol in *N. coriiceps*.

The acetate concentration was significantly reduced in warm-acclimated samples, while inosine content was elevated in both notothenioids. However, the total inosine concentration (mean concentrations of 0.031 μmol/g fresh weight in *N. rossii* and 0.059 μmol/g fresh weight in *N. coriiceps* under control conditions) was very low in gill tissue extracts compared to most other detected metabolites (e.g. Glucose: 0.198 μmol/g fresh weight in *N. rossii* and 0.143 μmol/g fresh weight in *N. coriiceps*, respectively).

While several metabolite concentrations in gill tissue extracts of *N. rossii* and *N. coriiceps* showed the same trends during warm-acclimation, some differences in the response to elevated temperatures were detected.

Supervised PLS-DA analysis provided evidence for a possible

Table 1
List of compounds identified in the ^{1}H–NMR spectrum of the gill tissue extract of *Notothenia rossii* and *Notothenia coriiceps* and corresponding chemical shifts. (Multiplicity s: singlet, d: doublet, t: triplet, m: multiplet).

Metabolite	Chemical shift
Amino acids	
Alanine	1.48 (d), 3.78 (m)
Glutamate	2.05 (m), 2.13 (m), 2.35 (m), 3.76 (m)
Glutamine	2.12 (m), 2.15 (m), 2.43 (m), 2.46 (m)
Glycine	3.56 (s)
Histidine	7.06 (s), 7.97 (s)
Isoleucine	0.94 (t), 0.99 (d)
Leucine	0.95 (d), 0.97 (d)
Methionine	2.14 (s)
Phenylalanine	7.33 (m), 7.36 (m), 7.43 (m)
Tyrosine	6.90 (d), 7.22 (d)
Valine	0.97 (d), 1.05 (d)
Organic osmolytes	
Betaine	3.87 (s)
Dimethylamine	2.72 (s)
Taurine	3.26 (t), 3.43 (t)
Trimethylamine N-oxide	3.27 (s)
Energy metabolism	
ADP/ATP	4.58 (m), 6.15 (d), 8.27 (s), 8.52 (s)
Creatine/ − phosphate	3.04 (s), 3.94 (s)
Glucose	4.65 (d), 5.24 (d)
Lactate	1.33 (d)
NADH/NADPH	5.98 (d), 6.21 (d), 8.23 (s), 8.46 (s)
UDP-glucose	5.98 (d), 5.99 (d), 7.94 (d)
Krebs cycle intermediates	
Citrate	2.54 (d), 2.70(d)
Succinate	2.41 (s)
Phospholipid related compounds	
Choline	3.19 (s)
Ethanolamine	3.13 (m), 3.81 (m)
myo-Inositol	3.54 (m), 3.63 (m), 4.06 (m)
O-Phosphocholine	3.21 (s)
sn-Glycero-3-phosphocholine	3.23 (s)
Miscellaneous	
Acetate	1.91 (s)
Anserine	3.78 (s)
Dimethyl sulfone	3.16 (s)
Glutathione	2.52 (m), 2.56 (m), 2.99 (m)
Hypoxanthine	8.19 (d), 8.21 (d)
Inosine	6.09 (d), 8.24 (s), 8.35 (s)
Malonate	3.12 (s)
Putrescine	1.77 (m), 3.05 (m)
Sarcosine	2.73 (s)

functional differentiation of both notothenioid species acclimated to 5 °C. Fig. 4 illustrates this segregation marked by the ellipses representing the 95% confidence interval.

A general pattern of metabolite responses in gill tissues was that in warm-acclimated *N. rossii* more amino acids displayed significantly lower concentrations than in *N. coriiceps* (see Table 3). Although the absolute concentrations of all these metabolites were lowered in *N. coriiceps* as well, these changes did not prove to be significant, except for glycine. Finally, the Krebs cycle intermediate succinate increased significantly in gill tissue samples collected from the 5 °C acclimated animals in *N. rossii*, but not in those from *N. coriiceps* (see Fig. 5).

4. Discussion

Aim of this study was to discover changes and differences of the metabolic profiles of two closely related Antarctic notothenioids, the Antarctic rock cod *Notothenia rossii* and the Antarctic yellow rock cod *Notothenia coriiceps* after three months incubation at 5 °C. This degree of warming has been projected for Antarctic surface waters around King George Island in the year 2100. The study focuses on major patterns, especially in the amino acid metabolism, and cannot explain all discovered differences in detail, because of the complexity of the total metabolic profile.

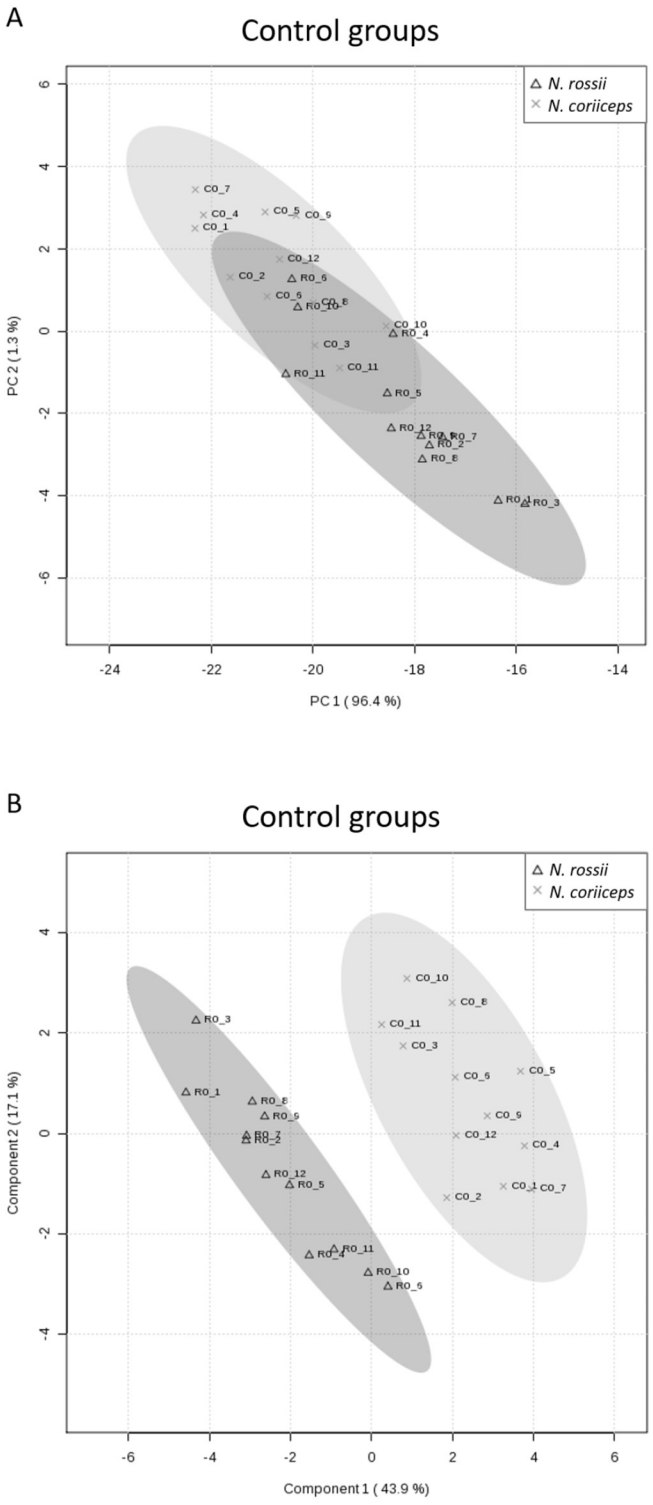

Fig. 2. A. Principal component analysis (PCA) of the normalised concentrations of assigned metabolites in the control groups acclimated to 0 °C.
B. Score Plot of the PLS-DA model for the normalised concentrations of assigned metabolites from gill tissue extracts in control groups of the two Antarctic fish species acclimated to 0 °C. Ellipses correspond to a confidence interval of 95% for each group.

4.1. Identified metabolites

The gill tissue is a metabolically active tissue requiring 7% of a fish's

Table 2
Metabolites of gills showing significant change in the normalised concentration values in *Notothenia coriiceps* (NC) in comparison to *Notothenia rossii* (NR) (control groups, acclimated to 0 °C).

Amino acids				
Methionine	NR	>	NC	$P < 0.05$
Phenylalanine	NR	>	NC	$P < 0.01$
Tyrosine	NR	>	NC	$P < 0.001$
Organic osmolytes				
Dimethylamine	NR	>	NC	$P < 0.01$
TMAO	NR	<	NC	$P < 0.001$
Energy metabolism				
Creatine/phosphate	NR	>	NC	$P < 0.001$
Glucose	NR	>	NC	$P < 0.001$
NADH/NADPH	NR	>	NC	$P < 0.001$
Citrate	NR	>	NC	$P < 0.05$
Phospholipid related compounds				
Choline	NR	>	NC	$P < 0.001$
Glycerophosphocholine	NR	>	NC	$P < 0.01$
Miscellaneous				
Acetate	NR	>	NC	$P < 0.01$
Inosine	NR	<	NC	$P < 0.05$
Sarcosine	NR	>	NC	$P < 0.001$

P-values determined using t-test and Mann-Whitney Rank Sum test, if prior Equal Variance test failed.

Table 3
Gill metabolites showing significant changes after acclimation from 0 to 5 °C in each species. Significance levels were analysed using normalised concentrations.

N. rossii			*N. coriiceps*		
Amino acids					
Glutamate	↓	$P < 0.05$			
Glycine	↓	$P < 0.05$	Glycine	↓	$P < 0.05$
Histidine	↑	$P < 0.05$			
Leucine	↓	$P < 0.01$			
Methionine	↓	$P < 0.05$			
Tyrosine	↓	$P < 0.001$			
Organic osmolytes					
Betaine	↓	$P < 0.001$			
			Dimethylamine	↓	$P < 0.05$
TMAO	↓	$P < 0.001$	TMAO	↓	$P < 0.001$
Energy metabolism					
Succinate	↑	$P < 0.05$			
Phospholipid related compounds					
Choline	↓	$P < 0.05$	Choline	↓	$P < 0.05$
Glycerophosphocholine	↓	$P < 0.01$			
myo-Inositol	↓	$P < 0.001$	myo-Inositol	↓	$P < 0.01$
Miscellaneous					
Acetate	↓	$P < 0.001$	Acetate	↓	$P < 0.01$
			Anserine	↓	$P < 0.01$
			Dimethylsulfone	↑	
Inosine	↑	$P < 0.001$	Inosine	↑	$P < 0.01$
Putrescine	↓	$P < 0.001$			
Sarcosine	↓	$P < 0.05$			

P-values determined using t-test and Mann-Whitney Rank Sum test, if prior Equal Variance test failed.

total oxygen demand, which is met by the direct oxygen uptake of gill filaments (Mommsen, 1984). Other important functions are osmoregulation, acid-base regulation and excretion of nitrogenous waste (Mommsen, 1984). All metabolite classes used in normal cell metabolism, such as sugars, proteins, nucleotides and lipids are present in gill tissues. Additionally, metabolites occur that are necessary to fulfil specific functions, such as osmolytes or glycoproteins.

The spectrum obtained from *N. rossii* (Fig. 1) was dominated by signals of osmolytes, such as taurine and TMAO, and free amino acids, such as alanine, glutamate and glycine. This finding is in line with ^{1}H–NMR studies on gill tissue extracts of other marine organisms, which also reported these metabolite groups as the most dominant (e.g. Tikunov et al., 2010; Liu et al., 2011; Xu et al., 2015).

Metabolites were grouped in different classes in order to identify major trends in cell metabolism.

1. The free amino acids: alanine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, tyrosine and valine found in both notothenioids were also those identified in

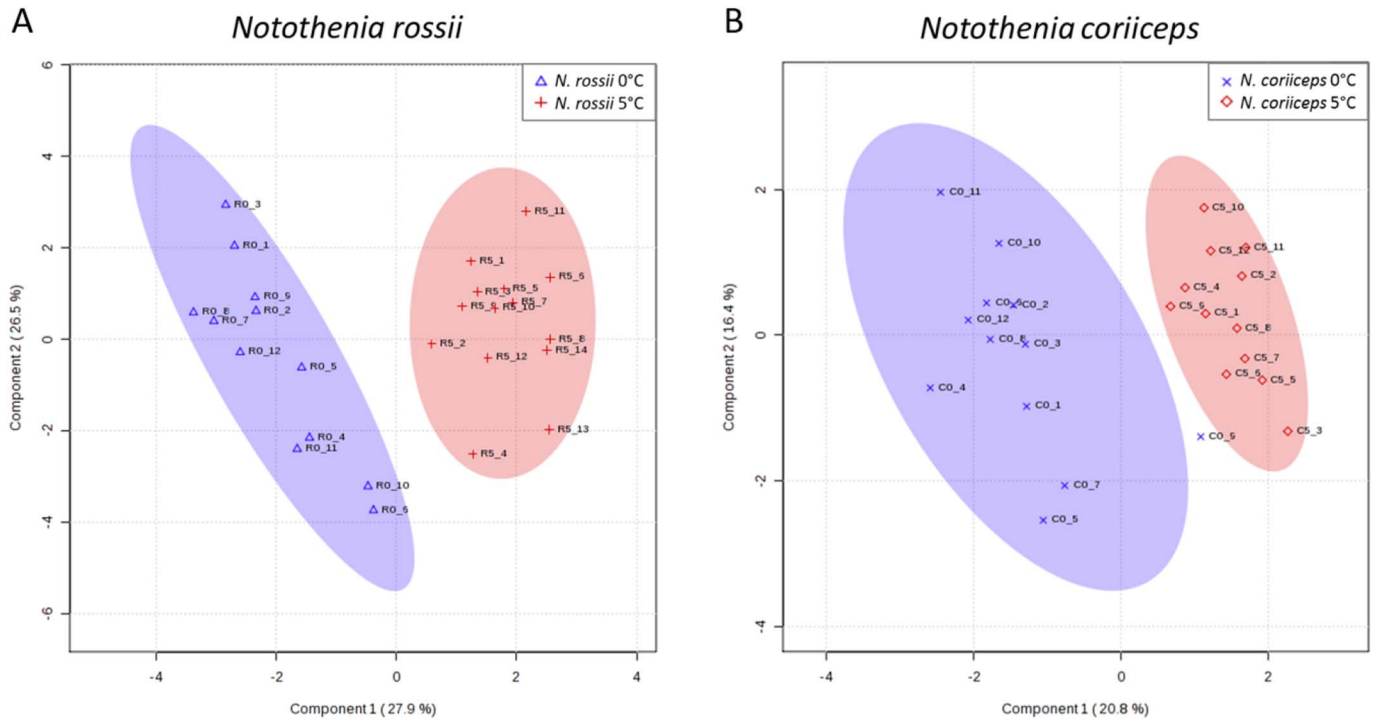

Fig. 3. Score Plot of the PLS-DA model for the normalised concentrations of assigned metabolites of gill tissue extracts of *Notothenia rossii* (A) and *Notothenia coriiceps* (B) acclimated to 0 °C and 5 °C. Ellipses correspond to a confidence interval of 95% for each group.

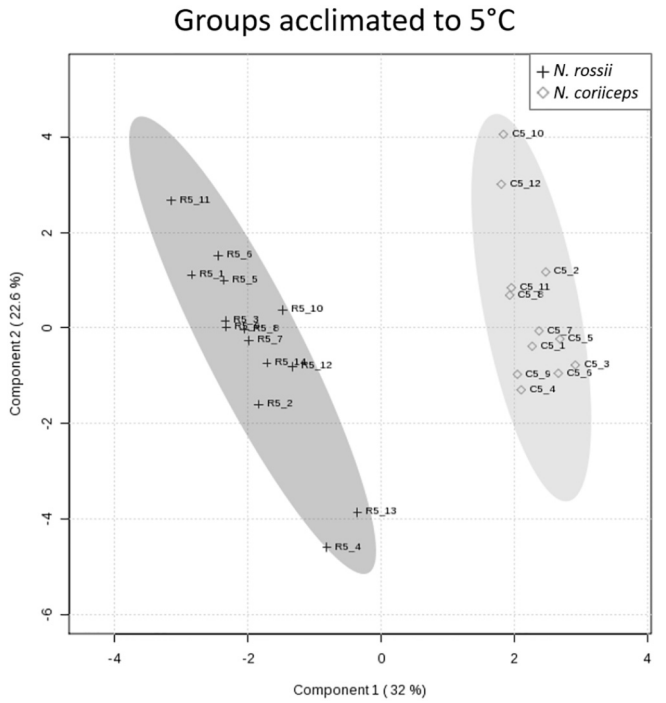

Fig. 4. PLS-DA Score plot for the normalised concentrations of assigned metabolites from gill tissue extracts of notothenioids acclimated to 5 °C. Ellipses correspond to a confidence interval of 95% for each group.

^{1}H–NMR spectra of other marine fish and mussel gill tissues (Tikunov et al., 2010; Xu et al., 2015). Besides being used in protein and keton synthesis, such amino acids are used as an energy source through oxidation. The rate of amino acids catabolism is usually high in fish, a finding related to a protein-rich diet (Mommsen, 1984). Nevertheless, investigations on aerobic metabolism in tilapia and toadfish revealed that gill cells in both species oxidise glucose and lactate at even larger rates than alanine or oleate (Perry and Walsh, 1989). Furthermore, amino acids are used for gluconeogenesis, lipogenesis and can act as osmolytes (Ballantyne, 2001; Yancey, 2005).

In the present study the highest concentrations were found for alanine and glutamate, which are described as good substrates for gluconeogenesis (Van Waarde, 1983).

2. Metabolites related to energy metabolism/Krebs cycle intermediates: glucose and lactate are used in oxidative metabolism as main energy source. These two and other assigned metabolites play major roles in various pathways of energy metabolism, such as creatine and phosphocreatine and the nucleotide derivatives ADP, ATP, NADH, NADPH. All metabolites are commonly present in ^{1}H–NMR spectra of various tissues from ectothermic animals (Mannina et al., 2008; Nestor et al., 2010; Tikunov et al., 2010; Lardon et al., 2013; Koyama et al., 2015).

Since the gills are a highly oxidative tissue, the Krebs cycle intermediates citrate and succinate were detected in gill tissue extracts, as shown in other studies (Lannig et al., 2010; Liu et al., 2011; Zhang et al., 2011).

Acetate is another metabolite that plays a decisive role in the metabolism of cells, as acetyl-CoA in the Krebs-cycle or as a precursor of lipids (Dean, 1969). Acetate was also found in aqueous gill extracts of goldfish (Xu et al., 2015).

3. Structural compounds: choline, inositol and ethanolamine are head groups for phospholipids and thus play a major role in the structural composition of membranes, as do the metabolites phosphocholine and glycerophosphocholine (Tocher et al., 2008). These essential structural components occur in almost all cells and were found in many gill tissues, for example in goldfish (Xu et al., 2015) and clams (Koyama et al., 2015).

4. Osmolytes: marine fishes actively regulate the osmotic gradient by active ion excretion across the gills and use small organic osmolytes as compatible solutes to maintain the intracellular homeostasis. Organic osmolytes have further functions, such as metabolic protection or stabilisation of macromolecules (Yancey, 2005). Among the osmolytes detected in gill tissue extracts were the methylamines TMAO, betaine and DMA, which are able to enhance protein folding and ligand binding. The dipeptide taurine has antioxidant and membrane stabilising activities (Roysommuti et al., 2003; Yancey, 2005). Taurine and TMAO occurred in exceptionally high concentrations in gill tissues of both notothenioids. While taurine is reported to occur in many organisms and different tissue types (Solís et al., 1988; Miller et al., 2000; Yancey, 2005; Kabli et al., 2009), high amounts of TMAO were found in several marine as well as Antarctic teleost fish. Antarctic fish have a higher osmolality than temperate fish, possibly reflecting cold adaption. High amounts of TMAO contribute to this high osmolality (Raymond and DeVries, 1998).

5. Miscellaneous metabolites: Several other detected metabolites with miscellaneous functions have been identified in other studies of aqueous gill extracts as well, such as the antioxidant glutathione and the purine derivatives inosine and hypoxanthine (Xu et al., 2015). Malonate was also found in gills of Manila clam (Zhang et al., 2011) and putrescine in gills of the clam *Corbicula japonica* (Koyama et al., 2015). In addition, some signals could not be assigned to a specific metabolite. For example, the signal at 3.35 ppm might be attributed to scyllo-inositol (Michaelis et al., 1993), that functions as a compatible osmolyte in deep-sea animals (Yancey, 2005). Further studies using analytical methods, such as mass spectrometry techniques or Fourier transform infrared spectroscopy (Dunn and Ellis, 2005) are necessary to characterise this metabolite and the other unknown compounds in order to gain knowledge about their functional role in cells.

The absolute metabolite concentrations determined from ^{1}H–NMR spectra in the gill tissues of both species are well in the range of concentrations reported in the literature, indicating the reliability of the used technique. Picone et al. (2011) found comparable amounts of the amino acids leucine and glycine and other metabolites, such as hypoxanthine and inosine, in muscle extracts of Gilthead sea bream (*Sparus aurata*). In addition, similar concentrations of some of the amino and organic acids were reported in gill tissues of flatfish using ^{1}H–NMR spectroscopy and gas chromatography (Fan et al., 1993).

4.2. Differences in the metabolite profiles of the two Antarctic notothenioids

The similarity of the ^{1}H–NMR spectra of both Antarctic notothenioids may reflect the close relationship of the two notothenioids (Near et al., 2004; Near and Cheng, 2008). This makes them ideal candidates for a comparison of their metabolite profiles in association with the ecological diversification related to differences in their lifestyle (e.g. benthic versus bentho-pelagic).

For control animals, a functional differentiation of the two Antarctic notothenioids according to metabolite concentrations in gill tissue extracts is supported by the PLS-DA score plot (Fig. 2B). The associated differences in the metabolite concentrations are presented in Table 2 and show that *Notothenia rossii* has significantly higher concentrations of the amino acids methionine, phenylalanine and tyrosine. Phenylalanine is a precursor of tyrosine and both are used for protein synthesis. *N. rossii* exhibited also higher concentrations in energy related metabolites and of phospholipid related compounds. On the other hand, *N. coriiceps* has significantly higher TMAO and inosine levels in gill cells, but less sarcosine.

The higher amounts of metabolites associated with energy metabolism, such as NADH and NADPH or phosphocreatine, point to a higher energy turnover of *N. rossii* in comparison to *N. coriiceps*. This finding supports the more active lifestyle found in the bentho-pelagic living *N. rossii*. In contrast, *Notothenia coriiceps* uses only 5.7% of its

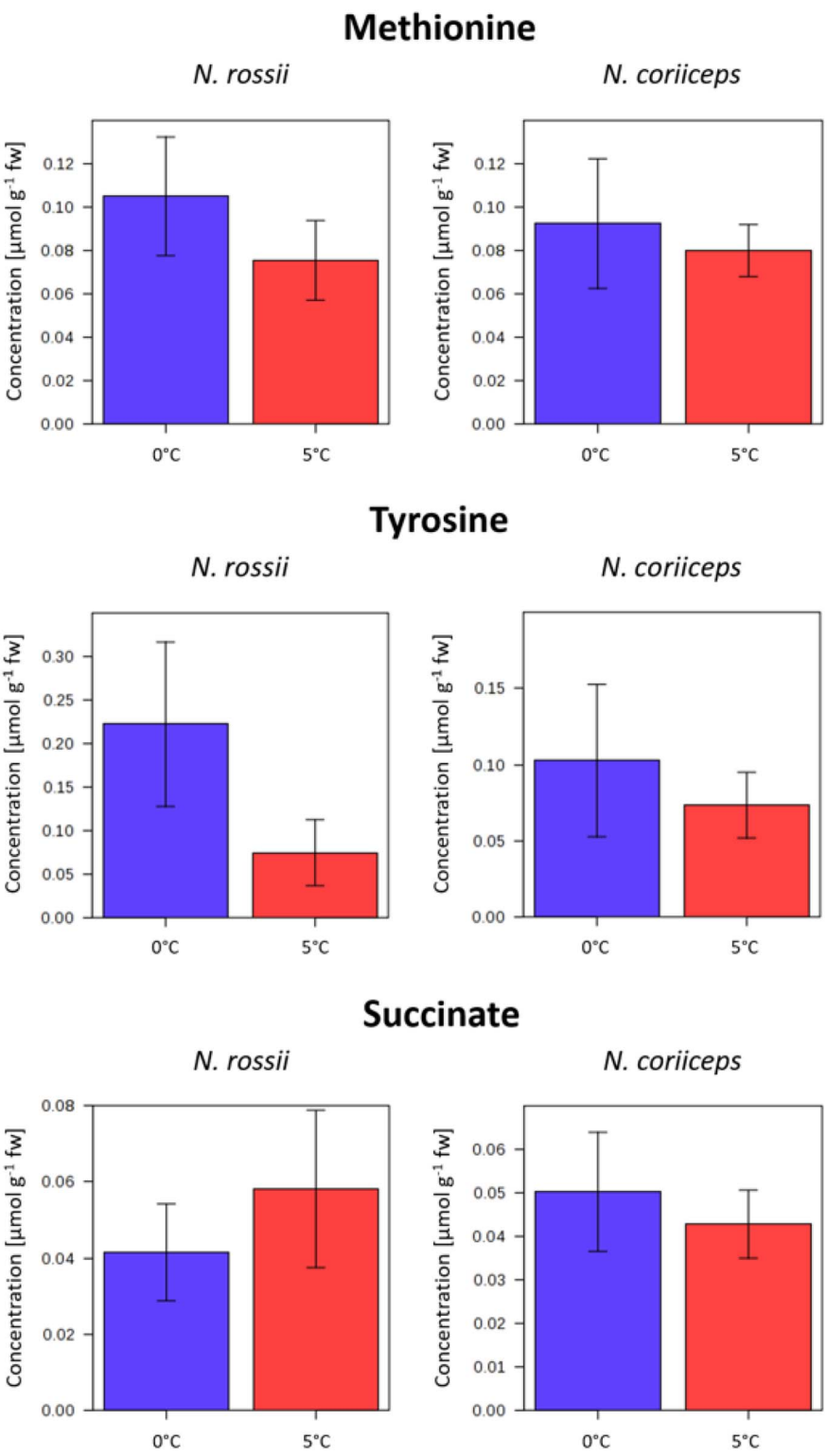

Fig. 5. Absolute concentrations of methionine, tyrosine and succinate that were significantly different in fish acclimated to 5 °C from those to 0 °C in *Notothenia rossii*, but not in *Notothenia coriiceps*. Significant changes were analysed using normalised metabolite concentrations. Note that the ordinates differ in scale.

total metabolic rate for activity and feeding (Campbell et al., 2007) and locomotory activity was detected for only 1.7% of a day (North, 1996). Additionally, it was suggested that *N. coriiceps* individuals from Potter Cove remain nearshore during their whole life cycle without migrating to offshore waters for feeding or breeding (N. Koschnick, personal communication). In contrast, *N. rossii* migrate offshore to feed and reproduce, which may come with a higher energy demand due to repeated active swimming (Casaux et al., 1990; Webb, 1971). This higher energy demand is reflected in a higher basal metabolism in *N. rossii* than in other less active notothenioids (Morris and North, 1984).

Among Antarctic notothenioids, which lack swim bladders, a trend is observed that more pelagic species have higher lipid contents and therefore also higher total amounts of phospholipids than benthic species (Clarke et al., 1984; Hagen et al., 2000). The higher concentration of choline and phospho-di- and mono-ester, which are used as head group for phospholipids, would support this hypothesis for the more pelagic *N. rossii*. Future studies on lipid composition, especially in lipid-rich compartments like liver or red muscles (Clarke et al., 1984)

are needed to confirm this.

The TMAO concentration was almost twice as high in *N. coriiceps* as *N. rossii*, while the concentration of DMA and another methylamine sarcosine, was significantly higher in *N. rossii*. Sarcosine has also been demonstrated to play a role as an organic osmolyte in some marine organisms (Van Waarde, 1988). TMAO is the major compatible solute typically found in many marine organisms including fishes and can also counteract the effect of hydrostatic pressure on enzyme function (Seibel and Walsh, 2002; and references herein). A varying intracellular composition of organic osmolytes might account for the observed differences in concentrations of these metabolites between both notothenioid species. Overall, the TMAO concentration is one to two orders of magnitude higher than of the other two osmolytes, so it is most unlikely fully compensated by the other metabolites. The accumulation of TMAO in marine organisms is still unclear and its specific role in notothenioids needs to be unravelled (Seibel and Walsh, 2002).

4.3. Acclimation processes to warming

The main changes observed in the metabolite concentrations of gill tissue extracts acclimated to 5 °C in comparison to the control group are a decrease in amino acids and a decline in metabolites related to lipid metabolism for both species. Reduced amounts of free amino acids were also found in long-term warm-acclimated Atlantic salmon (Kullgren et al., 2013), as were lipid related compounds. In addition, our results revealed lower concentrations of organic osmolytes in the warm-acclimated groups (Table 3). As expected, long term acclimation to elevated temperatures induced changes in amino acids in the gill tissue. The reduced cytosolic amounts of glycine in both species and the lower amounts of other amino acids in warm-acclimated *N. rossii* reflect an increased demand or turnover.

Amino acids can be used for protein synthesis and energy production by catabolism. While Kullgren et al. (2013) proposed a particular need for specific amino acids that were significantly lower in warm-acclimated Atlantic salmon, an increased overall demand for amino acids is also conceivable. Increasing protein damage at higher temperatures (Somero, 1995), especially in Antarctic fish with specific cold adapted protein synthesis mechanisms (Peck, 2016), might cause the higher demand for amino acids in the warm-acclimated notothenioid group in order to synthesise new functional proteins compensating for the damage. The decreased amino acids might even be related to the synthesis of heat-shock proteins, which were described as a nearly universal cellular response to elevated temperatures. These proteins stabilise other functionally relevant proteins, prevent aggregation of denatured proteins and help denatured proteins to re-fold into functional states (Feder and Hofmann, 1999). Despite the universal finding of the heat shock response (HSR), for example in notothenioids from New Zealand, there was no evidence for a heat-induced HSR in other Antarctic notothenioids (Hofmann et al., 2000; Hofmann et al., 2005). As there is no simple rule for possession and expression or loss of the classical heat shock response in Antarctic marine organisms (Clark and Peck, 2009), further studies need to address the question, whether some Antarctic species are able to show a HSR at elevated water temperatures. However, overall lower growth rates, which were found in long-term warm-acclimated Antarctic eelpout (Windisch et al., 2014), come along with lower rates of protein biosynthesis.

Besides the obvious role of amino acids in protein synthesis, free amino acids can also fuel the carbohydrate metabolism at higher temperatures. The degradation of branch-chain amino acids (BCAA), such as isoleucine, leucine and valine, leads to succinyl CoA, an intermediate of the tricarboxylic acid (TCA) cycle and glucogenic compounds that facilitates gluconeogenesis (Harvey and Ferrier, 2011). Those branched amino acids occur in lower concentrations in both species at warmer temperatures (data not shown, see Supplementary S3), which might be the result of a metabolic shift to carbohydrate-based metabolism, as indicated in the Antarctic eelpout to warming (Windisch et al., 2014). In the same study Windisch et al. proposed the importance of the glycine metabolism at low temperatures, since a strong temperature dependency in the expression of the glycine cleavage system was found in Antarctic eelpout. Indeed, glycine concentrations were reduced in both species acclimated to 5 °C. Whether the glycine cleavage system serves at low temperatures the catabolism of excessive glycine or rather its anabolism needs still to be investigated in future studies.

In the gill extracts the decrease in amino acids is more pronounced in *N. rossii*, which indicates a higher demand at 5 °C in comparison to *N. coriiceps*.

During thermal acclimation, the concentration of choline and myo-inositol in gill tissue were significantly decreased. In addition, a significant decrease of glycerophosphocholine was observed in *N. rossii*. These metabolites are used as structural components of phospholipids and can therefore be related to membrane changes. A decrease of the head groups in the cytosolic fraction might point to an elevated production of phospholipids and membrane elements. This explanation was suggested by lower choline concentrations found in warm-acclimated Atlantic salmon (Kullgren et al., 2013) and was also observed after long term acclimation to warming in the brain tissue of two gadid fish species (Schmidt et al., 2017). The increased building of structural membrane components is in line with the concept of homeoviscous adaptation, which states that membrane structures are remodelled at altered temperatures to compensate for harmful changes in the fluidity of membranes. During cold adaption more unsaturated fatty acids are incorporated in membranes to keep them fluid even at cold ambient temperatures. With rising temperatures, the membrane will become too disordered and will lose its integrity (Moyes and Ballantyne, 2011). The preferential synthesis of phosphocholine compared to phosphoethanolamine at higher temperatures discovered by Hazel and Williams (1990) is also reflected in our observations. While the amount of choline in the cytoplasm decreased significantly, ethanolamine concentrations did not change in the 5 °C acclimated groups. The increased synthesis of gill membranes in the warmth might also favour a better gas exchange due to increased surface area of gill cells. This could enhance the oxygen supply capacity of the gill tissue to enhance oxygen supply at rising temperatures (Allen, 1955; Pörtner and Peck, 2010).

While amino acids and phospholipid metabolism components change, the glucose concentration remained stable in all gill extracts. This is congruent with the observed steady state concentration of glucose in the plasma of Antarctic notothenioids (*Pagothenia borchgrevinki* and *Trematomus bernacchii*) acclimated to 4 °C (Lowe and Davison, 2005). Organic osmolytes concentrations, such as TMAO, were reduced in gills after long-term acclimation to higher temperatures. This was also reported for fish brain upon warming (Schmidt et al., 2017). The high osmolarity usually found in cold adapted fish for reducing the freezing point (Scholander et al., 1957) is decreased upon warming, resulting in the observed decrease in the organic osmolytes TMAO and myo-inositol, which serve as cryo-protectants in cold environments (Treberg et al., 2002; Vesala et al., 2012). In addition, a higher activity of ion transporters like the sodium-potassium ATPase at warmer temperatures will lead to decreased organic osmolytes. Strobel et al. (2012) observed a decreasing serum osmolarity in notothenioids due to warm acclimation. Intracellular osmolarity is then obtained by adjusting inorganic ions and the amount of organic osmolytes (Hochachka and Somero, 2002).

4.4. Differences in the acclimation capacities of the two notothenioid species

While both notothenioid species shared significant changes in their metabolite profiles due to higher temperature, Table 3 illustrates that *N. rossii* showed overall more significant changes than *N. coriiceps*. Especially the concentration of amino acids, such as glutamate, methionine, leucine or tyrosine were lower in *N. rossii* acclimated to 5 °C than in its sister species. Glycine was the only amino acid that decreased in both species, which might be due to the specific importance

of glycine at low temperatures, as proposed by its role in the glycine cleavage system (Windisch et al., 2014). In *N. rossii*, the lower levels of other amino acids in the cytosol and thus the resulting higher demand for substrate, emphasise the above-mentioned overall need for more amino acids. The amino acids are most likely used for energy production by catabolism in the gills (see above), which suggests a higher thermal energy demand of *N. rossii* in comparison to *N. coriiceps*. Klein et al. (2017) reported higher activities of enzymes involved in reactive oxygen detoxification and glutathione production in gill tissue of *N. rossii* in order to keep levels of oxidative damage similar to those observed in the rockcod *N. coriiceps*. The maintenance of high enzyme activities comes along with energetic costs and would be another factor influencing the performance capacities of *Notothenia rossii*.

The significantly higher amount of succinate in warm-acclimated *N. rossii* reinforce this hypothesis. Succinate can indicate mitochondrial anaerobiosis (Grieshaber et al., 1994). The onset of anaerobiosis leads to time limited survival of organism at critical temperatures (Pörtner, 2001). Succinate was shown to accumulate in tissues exposed to temperatures above the critical temperature in crustaceans (Frederich and Pörtner, 2000) and was also found to accumulate in Antarctic eelpout or Antarctic bivalves at critical temperatures (Pörtner et al., 1999; van Dijk et al., 1999).

The accumulation of succinate indicates a narrower temperature window of *N. rossii* than *N. coriiceps* where some metabolic processes in gills have reached their limit already at 5 °C. This assumption is supported by the finding that among cold-adapted notothenioids *Notothenia rossii* has only a moderate scope for acclimation and tolerance towards ocean acidification and warming. Strobel et al. (2013a, 2013b) proposed poor acclimation capacity based on routine metabolic rate, mitochondrial respiration capacities and mitochondrial enzyme activities of long-term acclimated individuals reflecting whole animal performance. In addition, uncompensated mitochondrial respiration rates that may reflect a high oxygen and metabolic demand at tissue level, have been found in *N. rossii* acclimated to 7 °C, while the more sub-Antarctic notothenioid *Lepidonotothen squamifrons* have shown partial compensation of mitochondrial respiration rates (Strobel et al., 2012; Strobel et al., 2013a). Taken together the observed changes in metabolite concentrations and the shifts in metabolic performance detected in other studies (Strobel et al., 2012, 2013a, 2013b; Klein et al., 2017) point to a higher thermal sensitivity of *N. rossii* compared to *N. coriiceps*.

5. Conclusions

In conclusion, the two Antarctic notothenioid species *Notothenia rossii* and *Notothenia coriiceps* are able to survive at temperatures about 3 °C higher than their ambient summer habitat temperatures. However, based on this metabolic approach and previous studies *N. rossii* seem to be more sensitive to warming than *N. coriiceps*. Untargeted NMR based metabolic profiling revealed significant changes in metabolite concentrations in gill tissue after long-term acclimation to elevated temperatures, while the metabolite composition remains unchanged in both species. The observed decrease in the amino acid concentrations was related to an enhanced catabolism in the gills, a finding that was also seen in brain tissues of polar fish. This might have, together with an increased protein degradation and the higher energy demand for restructuring cellular membranes, fatal consequences for notothenioids in the near future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2017.12.012.

Ethical approval

The experiments conducted were in accordance with the ethical standards of the federal state of Bremen, Germany, and were approved under reference number 522-27-11/02-00 (93).

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